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Comparison of Apolipoprotein A-I Values Assayed in Lyophilized and Frozen Pooled Human Sera by a Non-Immunochemical Electrophoretic Method and by Immunoassay

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Summary: Various immunochemical assays are used for measuring the apolipoprotein A-I in human serum, but results obtained by these assays often cannot be compared owing to methodological problems, the lack of reference methods and inadequate standardization. The electrophoretic apolipoprotein A-I assay avoids the problems associated with antibody-antigen interaction. The method involves spectrophotometric measurement of the Coomassie blue eluted from the apolipoprotein A-I bands of human serum after electrophoresis in a gradient gel. The assay was linear from 0.5 to 4.0 micrograms of apolipoprotein A-I. Within-assay variability was 3.2% and between-assay variability was 5.9%. Overall analytical recovery was 98%. The electrophoretic assay is suitable for the quantitation of apolipoprotein A-I in fresh, frozen or lyophilized serum pools. Analysis of 6 frozen serum pools showed a good correlation between this assay and an immunoturbidimetric assay ($r = 0.96$), and between this assay and consensus values ($r = 0.99$). Consensus values were derived from the results of different immunochemical assays performed in 6 laboratories in 3 different countries within 3 years. The mean apolipoprotein A-I concentrations in 3 lyophilized serum pools were 1.04 ± 0.02, 0.86 ± 0.02, and 0.45 ± 0.03 g/l (mean ± SD) by electrophoretic assay. The consensus values for these pools were 1.04, 0.86, and 0.43 g/l, respectively, as derived from the data of different immunochemical assay measurements performed in 84 laboratories in 34 various countries. The electrophoretic assay has been proposed as an alternative to the immunochemical assays for assigning the target values to reference and control materials, using pure and well-characterized apolipoprotein A-I as a primary standard.

Introduction

Apolipoprotein A-I accounts for 60–70% of the total HDL protein (1). High concentrations of plasma apolipoprotein A-I have been correlated with a reduced risk of premature cardiovascular disease (2).

Various isotopic and non-isotopic immunoassays are used in research and clinical laboratories for measuring apolipoprotein A-I in human serum or plasma. Immunochemical studies of apolipoprotein A-I have shown that purified apolipoprotein A-I and the apolipoprotein A-I in HDL differ in their apparent immunoreactivity; this difference depends not only on the type of immunoassay, but is also affected by certain modifications of the same immunoassay. This has been observed with both polyclonal antisera (3) and monoclonal antibodies (4, 5), and is probably responsible for the need to denature serum, plasma, and lipoprotein samples before use in most immunoassays where purified apolipoprotein A-I is used as the standard (6). Furthermore, apolipoprotein A-I immunoreactivity with both polyclonal and monoclonal antibodies can be altered by conditions of sample storage (5). Use of a reference serum as a secondary standard was shown to minimize the interlaboratory and inter-method variations in the results for unknown samples obtained with various immunochemical methods, using a variety of antisera and

primary standards (7). Nevertheless, the problem remains of choosing a suitable, stable primary or secondary standard with accurately measured apolipoprotein A-I values.

Development of the modern apolipoprotein A-I immunochemical assays is overlapped by that of other non-immunochemical methods recently reported for the quantitation of apolipoprotein A-I in whole human serum or plasma (8–10). One of these, based on density gradient ultracentrifugation following by high performance liquid chromatography gel filtration is laborious, and is of questionable suitability for apolipoprotein A-I measurements in lyophilized serum samples (8). Two others are based on densitometric scanning (9) and densitometric scanning or spectrophotometric measurement of the Coomassie blue eluted from the apolipoprotein A-I bands (10) of electrophoresed whole serum or plasma.

Since frozen and lyophilized serum pools are usually used as calibrators, reference materials, and external quality-assurance materials for apolipoproteins, one aim of the present study was to examine the suitability of the modified electrophoretic technique used by us for apolipoprotein A-I quantitation in fresh, frozen and lyophilized serum pools. Furthermore, we compared the apolipoprotein A-I values assayed in frozen and lyophilized serum pools by the independent electrophoretic method with those obtained by "consensus" with various immunoassays.

Materials and Methods

Reagents

Acrylamide, bis-acrylamide, sodium dodecyl sulphate (SDS), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulphate, Coomassie R-250, bromophenol blue and N,N-dimethylformamide were purchased from Serva, Heidelberg, Germany. Bio-Rad Protein assay was from Bio-Rad Laboratories, Richmond, CA. *Folin & Ciocalteu*'s phenol reagent was from Sigma Chemical Co., St. Louis, MO.

Gel electrophoresis

Electrophoresis was performed in 18 cm × 16 cm × 1.5 mm gels according to a similar system reported by *Sakai* et al. (9) with a linear gradient gel of 15–20% acrylamide, using homemade apparatus. The stacking gel consisted of 3.75% acrylamide, 0.125 mol/l Tris-HCl, 1 g/l SDS, pH 6.8. A 1.5-mm-thick 17-well comb was used in all experiments. Resultant wells were 5.0 mm wide and 2.0 cm high, allowing up to 60 µl of sample to be applied per line. Sample preparation, electrophoresis, gel staining and destaining were performed as described (10). After gel destaining the apolipoprotein A-I bands were excised and placed into 3.0-ml glass screw-top vials containing 1.0 ml distilled water-dimethylformamide (1+1, by volume). Vials were heated at 90 °C in a heating block for 1 h with periodic mixing. The absorbance was measured at 590 nm in a disposable semimicro cuvette (Bio-Rad Laboratories, Richmond, CA) with a Spectronic-2000 spectrophotometer (Bausch and Lomb, USA) blanked against distilled water-dimethylformamide (1+1, by volume).

Other methods

Proteins were determined according to the method of *Lowry* et al. (11) and with the Bio-Rad Protein assay (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions, with bovine serum albumin (1.37 g/l, Bio-Rad) as standard in both cases. Western blotting was performed according to *Towbin* et al. (12). Immunoturbidimetric determinations of apolipoprotein A-I were performed according to *Rifai & King* (13) with the sheep antiserum to human apolipoprotein A-I from Boehringer Mannheim (Cat. No. 726478, Germany). Purified apolipoprotein A-I was prepared according to *Sigalov* et al. (14). Six frozen serum pools with graded concentrations of apolipoprotein A-I (ranging from ca. 1.0 to 2.0 g/l) were prepared according to *Kuchmak* et al. (15).

Standards

Calibration serum for apolipoprotein A-I determination was from Boehringer Mannheim (Cat. No. 837237, Germany). Lyophilized serum pools (LS1–LS3) were obtained from WHO Lipid Reference Centre (Institute for Clinical and Experimental Medicine, Prague, Czechoslovakia) with mean apolipoprotein A-I values of 1.04, 0.86, and 0.43 g/l, which had been assigned by consensus (16) from the results obtained by different immunochemical assays in 84 laboratories in 34 various countries.

Results

As shown in figure 1, electrophoresis of fresh, frozen, and lyophilized pooled sera under the described conditions revealed a major protein band corresponding to apolipoprotein A-I in its electrophoretic mobility. Data obtained by parallel immunostaining with sheep anti-human apolipoprotein A-I antibodies confirm that all the apolipoprotein A-I contained in serum migrates as single band (not shown). The absence of other human proteins co-migrating with apolipoprotein A-I was confirmed by extraction of HDL from whole serum with specific antibodies against apolipoprotein A-I, followed by electrophoresis.

Optimal elution conditions

Our kinetic studies showed that the Coomassie blue was completely eluted from the stained apolipoprotein A-I bands after the heating at 90 °C for 1 h in aqueous dimethylformamide (fraction 0.5). After Coomassie blue elution, the absorbance of the solution at 590 nm was constant up to 5 h and longer. In contrast, the use of other solvent such as a dimethyl sulphoxide instead of a dimethylformamide led to a gradual decoloration of the solution under the above conditions due to a possible interaction of dimethylsulphoxide with Coomassie blue.

Primary standard

Purified apolipoprotein A-I was used as a primary standard. Desalted and lyophilized apolipoprotein A-I (5 mg) was dissolved in 5 ml of phosphate-buf-

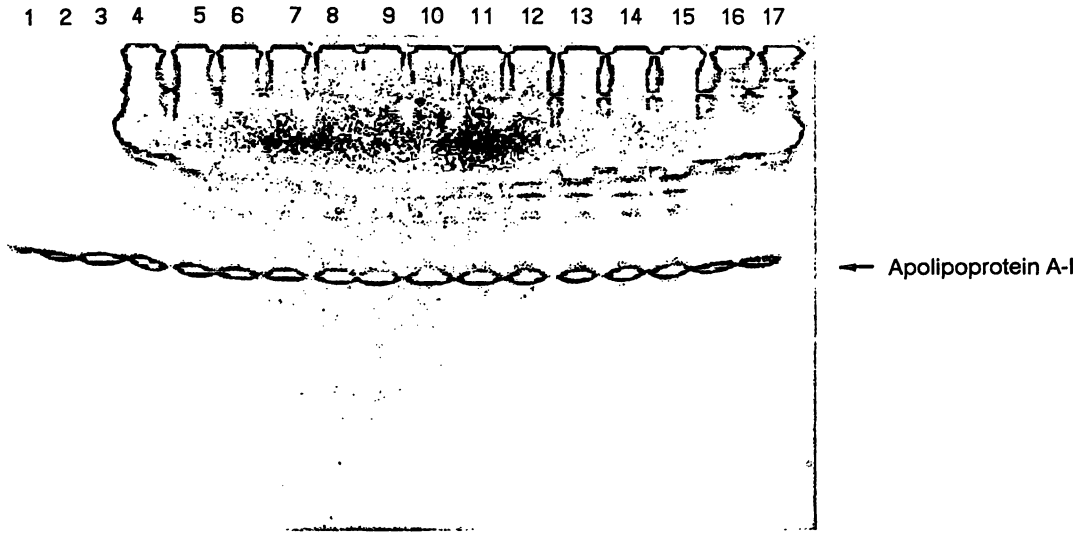

Fig. 1. Non-reducing SDS-gPAGE of 0.5, 1.0 and 2.0 µg apolipoprotein A-I (1—3), and fresh (4—7), frozen (8—13) and lyophilized (14—17) different serum samples.

fered saline (10 mmol/l Na_2HPO_4, 0.14 mol/l NaCl, pH 7.4) and its concentration was determined by the method of *Lowry* et al., the Bio-Rad Protein assay, and the present SDS-gPAGE (see Materials and Methods). The commercial reference solution of bovine serum albumin (1.37 g/l, Bio-Rad) was used as a standard in all three cases. The results obtained by these independent protein assays are given in table 1 (the data presented are the means ± SD of triplicate measurements by each method in 10 different experiments). As shown in table 1, the mean apolipoprotein A-I values obtained by both the method of *Lowry* et al. and SDS-gPAGE were not statistically different and were very similar to those expected. By contrast, the Bio-Rad Protein assay yielded significantly higher results ($P < 0.01$).

Tab. 1. Mean concentration of apolipoprotein A-I by different methods.

Apolipoprotein A-I, Mean ± SD[a] (g/l)		
Lowry et al.	Bio-Rad[b]	SDS-gPAGE
0.97 ± 0.02	1.12 ± 0.04[c]	1.02 ± 0.05

[a] The data presented are the means ± SD of triplicate measurements by each method in 10 different experiments.
[b] Bio-Rad, "Bio-Rad Protein assay".
[c] Significantly different from values obtained by the method of *Lowry* et al. and SDS-gPAGE ($P < 0.01$).

Precision

Repeated analysis of lyophilized pooled serum specimens at three different concentrations — 0.46, 0.86, and 1.04 g/l — gave within-run CVs ranging from 2.4% to 4.0% (n = 15). The between-run CVs for these concentrations ranged from 4.6 to 7.2% (n = 5).

Standard curves and linearity

Standard curves exhibited a correlation coefficient of 0.999 for absorbance at 590 nm of the Coomassie blue eluted from the apolipoprotein A-I bands (y) vs apolipoprotein A-I concentration (x): $y = 0.108x - 0.012$ g/l ($S_{yx} = 0.002$ g/l). The standard curve of purified human apolipoprotein A-I was linear from 0.5 to 4.0 micrograms.

Figure 2 depicts calibration curves developed with primary standard (isolated and purified apolipoprotein A-I) and three pooled serum samples in different forms: fresh, frozen and lyophilized. As shown, sample dilution yielded similar curves closely parallel to those of the primary standard, thus permitting the use of frozen or lyophilized sera as secondary standards.

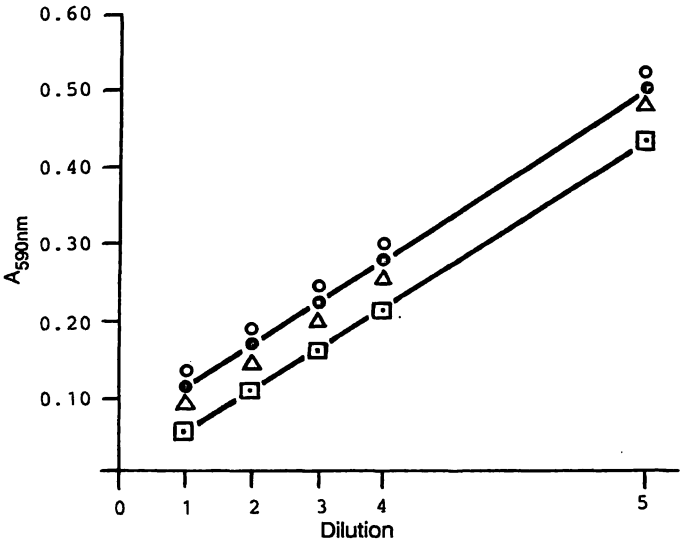

Fig. 2. Calibration curves prepared with three samples of the pooled serum, fresh (○), frozen (●) and lyophilized (△), and purified apolipoprotein A-I (⊡). The samples were diluted as follows: 1 : 29 (1), 1 : 14 (2), 1 : 9 (3), 1 : 6.5 (4) and 1 : 2.75 (5).

Analytical recovery and comparison methods

Analytical recoveries for apolipoprotein A-I ranged from 96 to 102% for lyophilized pooled serum; the starting concentration was 0.86 g/l, and this supplemented with extra purified apolipoprotein A-I (0.25, 0.5, and 0.75 g/l, n = 5 for each added amount of apolipoprotein A-I).

We also found that the mean apolipoprotein A-I values obtained for this serum sample by calibration with purified apolipoprotein A-I and using the technique of standard additions (17) were not statistically different as determined by *Student*'s paired-*t* test.

Comparison of apolipoprotein A-I concentrations measured in 30 serum samples by both SDS-gPAGE and the immunoturbidimetric assay yielded a good correlation (tab. 2); the mean values were not statistically different. We also compared the results obtained with the proposed method for six frozen serum-based materials containing graded concentrations of apolipoprotein A-I with those by immunoturbidimetrie assay, and with "consensus values" assigned by using the data of different immunochemical assays (radial immunodiffusion, electroimmunoassay, immunonephelometric and immunoturbidimetric assays) measured in 6 laboratories in 3 various countries within 3 years. As shown in table 2, we found a good correlation between these methods.

Tab. 2. Correlation between sodium dodecyl sulphate gradient polyacrylamide gel electrophoresis and immunoturbidimetric assay.

	Apolipoprotein A-I (x)		
	Fresh serum samples	Frozen serum pools[a]	
		Immunoturbidimetry	Consensus values[d]
y[b]	0.972x + 0.194[c]	0.906x + 0.202	1.068x − 0.282
n	30	6	6
r	0.932	0.961	0.990

[a] Six frozen serum pools with graded concentrations of apolipoprotein A-I.
[b] Apolipoprotein A-I by SDS-gPAGE.
[c] g/l.
[d] Consensus values: Apolipoprotein A-I values assigned by using the results of different immunochemical assay measurements performed in 6 laboratories in 3 various countries within 3 years. All correlations were significant ($P < 0.001$).

We also compared the mean apolipoprotein A-I values in 3 lyophilized serum pools measured by SDS-gPAGE with those assigned by using the data of different immunochemical assays performed in 84 laboratories in 34 various countries ("consensus values"). The results obtained were very similar (1.04 ± 0.02, 0.86 ± 0.02 and 0.45 ± 0.03 g/l by SDS-gPAGE, mean ± SD; and 1.04, 0.86 and 0.43 g/l by consensus, respectively).

Effect of freezing and lyophilization

A comparison of the apolipoprotein A-I values obtained from fresh serum pool samples with aliquots stored for one month at −70 °C (n = 5), and with lyophilized aliquots (n = 5, after correcting for dilution attributable to reconstitution) gave very similar results; the mean values were not statistically different (the dilution factor was determined by sodium quantification before and after lyophilization and was about 8%).

Discussion

It has been shown (7) that frozen and lyophilized serum-based reference, calibration and quality assurance materials are suitable for use with apolipoprotein A-I analyses. Two methods can be used to assign target apolipoprotein A-I values to these materials:

1) the assignation of "consensus reference values", using the results of different immunochemical assay measurements performed in a specially designed study by a "labeling reference group" of experts;
2) analysis by any suitable method used in conjunction with a well-characterized primary standard (i.e., purified apolipoprotein A-I) or international biological standards.

We describe here the development of a precise, specific, and reproducible technique utilizing SDS-gPAGE for direct apolipoprotein A-I measurement in human serum that does not depend on any immunoreaction and therefore avoids the problems associated with antibody-antigen interaction (the heterogeneity of antigenic sites in apolipoprotein A-I, heterogeneity in their expression, and heterogeneity of antibodies raised against apolipoprotein A-I). The assay is inexpensive and requires no radioisotopes, and the method possesses intrinsically high recovery, due to minimal sample manipulation. No special sample pretreatment is required, and serum samples subjected to freezing or lyophilization can be used without any statistically significant change in results. By using a dimethylformamide-water solvent system, the Coomassie blue elution procedure can be reduced to 1 h in contrast to 4 h for the two-phase *n*-butanol-water system (10).

Our data indicate that apolipoprotein A-I in native HDL particles in whole fresh, frozen or lyophilized serum, and purified apolipoprotein A-I are similarly quantified by SDS-gPAGE. Furthermore, the parallelism obtained in dilution curves (see fig. 2) lends validity to the use of frozen or lyophilized secondary standards (for example, international biological stan-

dards) as well as a primary standard, i.e., purified apolipoprotein A-I.

A highly positive, statistically significant correlation was noted between human apolipoprotein A-I values derived from SDS-gPAGE and immunoturbidimetric assay in fresh and frozen serum samples (see tab. 2). Moreover, the mean apolipoprotein A-I values measured by SDS-gPAGE in three lyophilized serum pools were similar to those obtained by the "consensus values" method. These observations lead us to suppose that the frozen and lyophilized serum-based materials with apolipoprotein A-I values assayed by SDS-gPAGE, using well-characterized apolipoprotein A-I as a primary standard, can be used as potential secondary standards for different immunoassays.

In this study, we used purified human apolipoprotein A-I dissolved in phosphate buffered saline (pH 7.4) as a primary standard to which a gravimetrically defined mass value (1 g/l) has been assigned. As shown in table 1, this value is similar to the apolipoprotein A-I values obtained with the method of *Lowry* et al. and with SDS-gPAGE. Since the mass of apolipoprotein A-I as measured by amino acid analysis is nearly identical to that determined by the *Lowry* method (18), one might conclude that bovine serum albumin can be used in our SDS-gPAGE system as a primary standard for apolipoprotein A-I determination in human serum. But further detailed studies are needed on both apolipoprotein A-I and bovine serum albumin chromogenicities with Coomassie blue R-250.

Finally, the SDS-gPAGE system described here can provide specific and reproducible determinations of apolipoprotein A-I in fresh, frozen and lyophilized human serum samples. The apolipoprotein A-I values assayed by the electrophoretic method may differ from those obtained by a particular immunoassay (for example, immunoturbidimetric assay), but they are nearly identical to "consensus values" calculated from the data obtained in different laboratories by various immunoassays. Thus, the electrophoretic assay can be readily applied as an independent assay for assigning accurate apolipoprotein A-I values to distributed serum-based reference, calibration and quality-assurance materials.

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